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(54) Title: OXYGEN ACTIVATABLE FORMULATIONS FOR DISINFECTION OR STERILIZATION			
(57) Abstract Methods and composition are disclosed for producing air-activated, i.e., oxygen activated, disinfectant-sterilent solutions. Solutions containing a haloperoxidase (i.e., a halide: hydrogen peroxide oxidoreductase, such as myeloperoxidase, eosinophil peroxidase or lactoperoxidase) plus a halide or combination of halides (i.e., chloride, bromide and/or iodide), an oxidase (i.e., a substrate: oxygen oxidoreductase) capable of generating hydrogen peroxide, and a substrate specific for that oxidase, are separately prepared under aerobic conditions, but all of the component solutions are made anaerobic prior to final combination and mixing. The anaerobic formulations are dispensed into containers capable of maintaining the anaerobic condition (e.g., pressurized canisters). Dispensing the solution at the time of use exposes the formulation to air (i.e., oxygen) which activates its disinfectant-sterilent properties.			

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OXYGEN ACTIVATABLE FORMULATIONS FOR DISINFECTION OR STERILIZATION

Field of the Invention

The present invention relates to methods and compositions for antiseptis,
5 disinfection or sterilization. More particularly, the present invention relates to
methods and compositions that remain inactive as packaged for storage, but become
active on exposure to air as an antiseptic, disinfection or sterilent agent when
dispensed for use.

Background of the Invention

10 Antisepsis is defined as substantial reduction of microbial content whereas
disinfection is the elimination of all life forms capable of causing disease. Practically,
disinfection implies destruction of all viable microorganisms except spores.
Sterilization means the complete elimination of all viable microorganisms including
spores (Hospital Infections, 2nd Ed. (Bennett, J.V. and Brachman, P.S. eds.), Little,
15 Brown and Co., Boston, MA, pp. 238-241, 1986). The acceptable methods of
sterilization in current use are autoclaving (steam under pressure), dry heat, and gas
sterilization (e.g., ethylene oxide). Sterilization by soaking in antiseptics is typically
incomplete and is indicated only in circumstances where the sterilization methods
described above are not applicable.

There are limitations to all of the sterilization methods described above. Many materials and devices are destroyed by dry heat or steam sterilization. Gas sterilization typically requires prolonged contact, e.g., exposure for greater than an hour, and a post-sterilization period for dissipation of the gas from the treated material. Lensed instruments or porous items typically require 24 to 48 hours of exposure to air before use. On the other hand, sterilization with germicidal agents, such as gluteraldehyde (2%), formaldehyde (8%)-alcohol (70%), or hydrogen peroxide (6%), requires exposure times ranging from 6 to 18 hours. These germicidal agents are also highly toxic and are indiscriminant in their toxic effect. As such, these sterilents cannot be brought in direct contact with host tissue, and have limited utility as sterilants for biomedical devices, such as contact lenses, medical and surgical instruments, and for wound cleaning. For example, an antimicrobial agent used to disinfect or sterilize a contact lens must possess a number of unique characteristics. On one hand, it must be effective against microorganisms which may be dangerous to the eye. At the same time, it must be tolerated in the delicate ocular environment of the user, and also not damage the contact lens itself. A number of contact lens disinfecting and preserving solutions are known in the art. Typically such solutions employ either sorbic acid, thimerosal, chlorhexidine, a polyquaternary germicide, a synthetic antibiotic or a conventional quaternary germicide, such as benzalkonium chloride. However, these conventional antimicrobial agents have drawbacks that tend to restrict their use. For example, sorbic acid characteristically contains formaldehyde residues, thimerosal in some patients acts as an allergy sensitizer, and chlorhexidine is relatively toxic. Also, a problem exists in that soft contact lens materials have a tendency to bind and concentrate antimicrobial agents and other active ingredients commonly found in contact lens care solutions, in some cases to hazardous levels. For example, benzalkonium chloride is typically not used with soft contact lenses due to its tendency to be taken up into the lens matrix. In addition, many of the antimicrobial agents known to date are relatively ineffective against a number of fungi and yeasts which are problematic in the ocular environment.

U.S. Patent No. 5,389,369 discloses an improved haloperoxidase-based system for killing bacteria, yeast or sporular microorganisms by contacting the microorganisms, in the presence of a peroxide and chloride or bromide, with a haloperoxidase and an antimicrobial activity enhancing α -amino acid. Although compositions and methods of U.S. Patent No. 5,389,369 have been found to be highly effective antimicrobials, the components must be separately stored and maintained in

order to prevent haloperoxidase/peroxide interaction and depletion prior to dispensing for use.

Therefore, there exists a need for methods and compositions for disinfecting and/or sterilizing materials or devices, such as contact lenses, surgical instruments and other biomedical devices, which is effective against bacteria, fungi and yeasts, which is tolerable by the user, which does not damage the devices, and which is designed for ease and convenience of storage and use. Ideally, such disinfectant-sterilent compositions should be fast acting with minimal host toxicity and maximal germicidal action. The compositions should be easy to deliver, should not damage the material or device treated, and should not cause damage to host tissue on contact. Depending upon the strength of composition and the time interval of exposure, the compositions should produce antiseptics, disinfection or sterilization.

Summary of the Invention

The present invention describes methods and compositions for producing air-activated, i.e., oxygen (O_2) activated, disinfectant-sterilent solutions. Solutions containing a haloperoxidase (i.e., a halide:hydrogen peroxide (H_2O_2) oxidoreductase, such as myeloperoxidase, eosinophil peroxidase or lactoperoxidase) plus a halide or combination of halides (i.e., chloride, bromide and/or iodide) in appropriate concentrations, an oxidase (i.e., a substrate: O_2 oxidoreductase) capable of generating H_2O_2 , and a substrate specific for that oxidase, are separately prepared under aerobic conditions, but all of the component solutions are made anaerobic prior to final combination and mixing. The anaerobic formulations are dispensed into containers capable of maintaining the anaerobic condition (e.g., pressurized canisters). Dispensing the solution at the time of use exposes the formulation to air (i.e., O_2) which activates its disinfectant-sterilent properties. O_2 is the rate limiting component for oxidase generation of H_2O_2 . Under aerobic conditions the oxidase catalyzes the oxidation of its substrate and the reduction of O_2 to generate H_2O_2 . In turn, H_2O_2 serves as substrate for haloperoxidase which catalyzes the oxidation of halide to hypohalous acid. Hypohalous acid reacts with an additional H_2O_2 to generate singlet molecular oxygen (1O_2). Hypohalous acid (e.g., hypochlorous acid) and especially 1O_2 are potent microbicidal agents. Both haloperoxidase generation of hypohalous acid and its reactive consumption to yield 1O_2 are dependent on the availability of H_2O_2 . A high rate of H_2O_2 generation does not result in the accumulation of hypohalous acid, but instead results in a high rate of 1O_2 production. The microbicidal capacity and toxicity of 1O_2 are limited by the half-life of this metastable electronically excited reactant, and as such, disinfectant-sterilent activity is temporally

defined by and confined to the dynamics of oxidant generation. Disinfectant-sterilent activity of a formulation requires air exposure and is dependent on the presence of the halide-haloperoxidase combination employed, the activity of the oxidase present, the availability of oxidase-specific substrate and the availability of O_2 .

5 For these disinfectant-sterilent formulations, O_2 is the essential and limiting component for microbicidal action. The formulation must contain sufficient haloperoxidase to produce the desired microbicidal effect. However, the relative concentrations of oxidase and its substrate can be adjusted to produce a broad spectrum of microbicidal activities ranging from rapid, high intensity microbicidal
10 action of short duration to slow and prolonged microbicidal plus sporicidal action. By increasing the oxidase and making the oxidase substrate concentration limiting, the formulation will rapidly convert substrate to H_2O_2 producing a highly potent but time-limited microbicidal action. Once the substrate is exhausted there is a cessation of oxidative activity. On the other hand, limiting the concentration of oxidase limits
15 the rate of H_2O_2 generation and produces a slow but sustained microbicidal action. The concentration of oxidase limits the rate of H_2O_2 production, and the concentration of substrate limits the quantity and duration of H_2O_2 production.

Haloperoxidases have a very high microbicidal capacity and low host toxicity. These characteristics, combined with the ability to formulate the temporal dynamics of
20 disinfectant-sterilent activity (i.e., the ability to regulate the time period or window of maximum microbicidal action) assure excellent chemical sterilent activity with minimum host toxicity. In the absence of substrates, haloperoxidases show no direct toxicity to mammalian cells. Haloperoxidase oxidation and oxygenation activities are functionally linked to the availability of H_2O_2 . As such, materials or devices (e.g.,
25 endoscopy tube) sterilized by these haloperoxidase formulations can be brought in direct contact with host tissue immediately following sterilization.

Detailed Description of the Preferred Embodiment

In accordance with the present invention, methods are provided for killing or inhibiting the growth of microorganisms comprising the steps of:

- 30 (a) maintaining under substantially anaerobic conditions a microbicidal composition comprising a haloperoxidase, a halide, and a peroxide generating agent capable of generating peroxide upon exposure to oxygen;
- (b) exposing the composition to oxygen to activate the microbicidal activity of the composition; and
- 35 (c) contacting the microorganisms with the activated composition to kill or inhibit the growth of the microorganisms.

The components are preferably prepared, combined and stored anaerobically (i.e., in the relative absence of oxygen). The anaerobic formulation remains inactive until exposed to air at the time of application. Air exposure provides oxygen, the rate limiting component for microbicidal action, to activate the formulation to kill or inhibit the growth of the microorganisms.

In another aspect of the present invention, hermetically sealed containers or packages are provided that maintain under substantially anaerobic conditions a formulation comprising a haloperoxidase, a halide, and a peroxide generating agent capable of generating peroxide upon exposure to oxygen. Means are provided for releasing the formulation from the container or package, whereby the formulation is activated upon exposure to air to enable killing or inhibition of the growth of microorganisms.

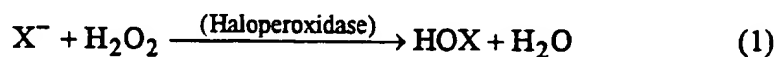
As used herein, the term "anaerobic" or "substantially anaerobic" means in the absence of oxygen or substantially in the absence of oxygen. Preferably, compositions of the invention are maintained until dispensed for use under substantially anaerobic conditions having less than about 1000 parts per million (ppm) of oxygen, more preferably less than about 500 ppm of oxygen, and most preferably less than about 250 ppm of oxygen.

Haloperoxidases useful in the present invention are defined as halide:hydrogen peroxide oxidoreductases (e.g., EC No. 1.11.1.7 and EC No. 1.11.1.10 under the International Union of Biochemistry) for which halide is the electron donor or reductant and peroxide is the electron receiver or oxidant. Any haloperoxidase which catalyzes the halide dependent generation of singlet molecular oxygen from hydrogen peroxide may be used in the present invention. Suitable haloperoxidases include myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), chloroperoxidase (CPO), and derivatives thereof, with the presently preferred haloperoxidases being myeloperoxidase and eosinophil peroxidase. By "derivatives thereof" as used herein generally means chemically or functionally modified MPO, EPO, CPO, and LPO which are capable of specifically binding to target microorganisms or specific eukaryotic cell types and which retain haloperoxidase activity in the enhancement of the disproportionation of peroxide to form singlet molecular oxygen in the presence of a suitable halide, as described herein.

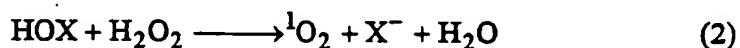
Suitable halides for use in the methods and compositions of the invention include bromide, chloride and/or iodide. The use, selection, and amount of halide employed in a particular application will depend upon various factors, such as the haloperoxidase used in the antiseptic composition, the desired antiseptic, disinfection

or sterilization effect, and other factors. When the haloperoxidase is MPO or CPO, the halide may be bromide or chloride. The amount of chloride employed will preferably fall in the range of about 10 μmol chloride to about 150 μmol chloride per ml of solution to approximate physiological conditions. When the haloperoxidase is EPO or LPO, chloride is relatively ineffective as a cofactor, and accordingly, the preferred halide is bromide. When included in liquid compositions, the compositions of the invention may comprise from about 1 nmol bromide to about 50 μmol bromide per ml of liquid composition, more preferably from about 10 nmol bromide to about 10 μmol bromide per ml of liquid composition, and most preferably from about 100 nmol bromide to about 1 μmol bromide per ml of liquid composition.

In the presence of sufficient halide, H_2O_2 is the rate limiting substrate for haloperoxidase microbicidal action. Microbicidal activity is linked to haloperoxidase generation of hypohalous acid:



and to the secondary generation of singlet molecular oxygen ($^1\text{O}_2$):



Both HOX and $^1\text{O}_2$ are potent antimicrobial reactants. Since H_2O_2 is required for HOX generation and H_2O_2 reacts with HOX to yield $^1\text{O}_2$, the haloperoxidase system guarantees that HOX generated will not accumulate but will further react to yield $^1\text{O}_2$, a metastable electronically excited molecule of potent reactivity but limited lifetime.

It is an important feature of the present invention that the haloperoxidase system components be maintained under anaerobic conditions until ready for use, and then be activated upon exposure to oxygen. This oxygen- or air-activated feature of the present invention results from including in the formulation an oxygen-dependent peroxide generating agent that produces peroxide on exposure to oxygen in the air. Suitable oxygen-dependent peroxide generating agents include any chemical system that can generate peroxide on exposure to O_2 , provided the system does not inhibit haloperoxidase function, does not damage the materials or devices to be disinfected or sterilized, and is not toxic to mammalian tissue at the concentrations employed. In a presently particularly preferred embodiment, the peroxide generating agent comprises: (a) an oxidase (substrate: O_2 oxidoreductase), and (b) a substrate specific for the oxidase. Oxidases are substrate-specific enzymes that generate H_2O_2 on exposure to O_2 , according to reaction (3):

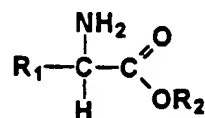


Since H_2O_2 production is dependent on both oxidase-specific substrate and O_2 , oxidases are particularly useful in the practice of the invention. Representative oxidases for this purpose (together with their respective substrates) include, but are not limited to, glycollate oxidase, glucose oxidase, galactase oxidase, hexose oxidase, cholesterol oxidase, aryl-alcohol oxidase, L-gulonolactone oxidase, galactose oxidase, pyranose oxidase, L-sorbose oxidase, pyridoxine oxidase, alcohol oxidase, L-2-hydroxyacid oxidase, ecdysone oxidase, choline oxidase, aldehyde oxidase, xanthine oxidase, pyruvate oxidase, oxalate oxidase, glyoxylate oxidase, pyruvate oxidase, D-aspartate oxidase, L-aminoacid oxidase, amine oxidase, pyridoxamine-phosphate oxidase, D-glutamate oxidase, ethanolamine oxidase, tyramine oxidase, putrascine oxidase, sarcosine oxidase, N-methylaminoacid oxidase, N-methyl-lysine oxidase, hydroxynicotine oxidase, glycerol-3-phosphate oxidase, nitroethane oxidase, acetylindoxyl oxidase, urate oxidase, hydroxylamine amine oxidase, and sulphite oxidase. Oxidases that generate free radical hydrodioxylic acid (HO_2) and its conjugate base superoxide (O_2^-) can also be employed; ultimately these radical intermediates disproportionate to yield H_2O_2 . When maintained under anaerobic conditions, the oxidase and its substrate are inactive because O_2 is unavailable to participate in the oxidase/substrate reaction (1). As such, no H_2O_2 is produced until the formulation is exposed to its rate limiting component, O_2 .

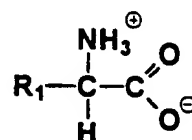
Agents capable of producing hydrogen peroxide on exposure to oxygen, e.g., peroxide producing oxidases, are also particularly useful for dynamic control of the amounts of hydrogen peroxide present at the site of antimicrobial activity. Such agents maximize antimicrobial activity of the composition by providing and maintaining a steady, low level concentration of H_2O_2 . Accordingly, the amount of such agents to be employed will be highly dependent on the nature of the agent and the effect desired, but will preferably be capable of producing a steady state level of from about 1 pmol to about 1 μmol of hydrogen peroxide per ml of liquid per minute, depending on the type and concentration of halide available at the site of antimicrobial activity. When the formulation is to be used as a disinfectant-sterilizing solution, the oxidase and its substrate can be adjusted to provide relatively high steady-state concentrations of H_2O_2 lasting for the required sterilization period. The disinfection-sterilizing action is terminated with exhaustion of the oxidase substrate or relative to the rate of oxidase degradation.

Optionally, the antimicrobial activity of the formulations of the invention against yeast and sporular microorganisms may be improved by including within the formulations a suitable antimicrobial activity enhancing agent, as disclosed in U.S. Patent No. 5,389,369, the disclosure of which is included herein by this reference.

- 5 Generally, suitable antimicrobial activity enhancing agents of the invention are agents that enhance the antimicrobial activity of the haloperoxidase antimicrobial system against yeast and sporular microorganisms by labilizing the yeast and spore forms of microorganisms to haloperoxidase microbicidal activity, and that do not produce adverse effects on the haloperoxidase activity of the system or undesirable effects in
10 the environment of use. Presently preferred activity enhancing agents of the invention include α -amino acid compounds of the formula:



- wherein R_1 is hydrogen, a straight or branched chain alkyl group having from 1 to 6 carbon atoms, or an unsubstituted or hydroxy or amino substituted straight or
15 branched chain arylalkyl group having from 7 to 12 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbon atoms. As used herein, amino acids may be in their acid form, as shown above, or may be in their zwitterionic form represented by the formula:



- 20 wherein R_1 and R_2 having the meanings set forth above, and may be in either *l*- or *d*-enantiomeric configurations. Representative alkyl R_1 groups include, for example, methyl, hydroxymethyl, isopropyl, 2-isobutyl, 1-isobutyl, hydroxy ethyl and amino butyl groups. Representative arylalkyl R_1 groups include, for example, tolyl and hydroxytolyl groups. Presently particularly preferred alkyl R_2 groups include methyl
25 and ethyl groups. Representative antimicrobial activity enhancing agents of the invention include α -amino acids selected from the group consisting of glycine and the *l*- or *d*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine and the alkyl esters thereof. The presently most preferred antimicrobial activity enhancing agents are glycine and *l*-alanine.

In accordance with other aspects of the invention, anaerobic formulations containing oxidase, oxidase-specific substrate, halide and haloperoxidase can be formulated and packaged for long-term storage at ambient temperature. Such formulations produce potent microbicidal action upon air exposure. Furthermore, O₂-activated disinfectant-sterilents can be formulated to achieve different degrees of potency and different temporal periods of activity. As can be deduced from equation (1), above, the rate of H₂O₂ generation is dependent on the concentration of oxidase, whereas the quantity of H₂O₂ generated is proportional to the quantity of substrate present. When substrate is not limiting, the rate of H₂O₂ generation is directly proportional to the oxidase activity. When oxidase activity is not limiting, the quantity and duration of H₂O₂ generation is dependent on the availability of oxidase-specific substrate. If a high potency, short lived disinfectant-sterilent action is desired, the formulation should have adequate haloperoxidase and halide, and a relatively high oxidase activity with substrate sufficient to confine activity to the desired time window. A lower potency but long-lived sterilent action can be formulated with adequate haloperoxidase and halide, relatively low oxidase activity, and sufficient substrate to sustain reaction for the temporal period desired. As shown in the following examples, systems of this type have been formulated to achieve sustained microbicidal-sporicidal action for a two-day period following air exposure.

In one particularly preferred embodiment, the methods and compositions of the invention are used as antiseptic agents exhibiting enhanced haloperoxidase antispore and antiyeast activity against a broad range of pathogenic microorganisms including bacteria and fungi. For use in contact with host tissue, the antiseptic systems are based on the use of dioxygenating haloperoxidase enzymes which exhibit selective affinity for pathogenic microorganisms. As such, high potency microbicidal action can be directed to the target microorganisms without associated host tissue destruction or disruption of normal flora; i.e., the antiseptic action is selective and confined to the target microorganism.

When properly formulated, haloperoxidase-enhancer preparations can be employed to disinfect and even sterilize materials and devices. High potency haloperoxidase-enhancer formulations can serve as *in vitro* disinfecting or sterilizing preparations. By limiting the time period of hydrogen peroxide availability, haloperoxidase-enhancer formulations can be made sufficiently potent to insure disinfection and even sterilization of a material or device before contact with host tissue. Any potential toxicity to normal flora and host tissue associated with the use of these high potency formulations will cease when peroxide is depleted, and as such,

the formulation-treated material or device can be brought in contact with host tissue without additional washing to detoxification.

In another embodiment of the invention, the compositions of the invention may be specifically designed for *in vitro* applications, such as disinfecting or sterilization of medical devices, contact lenses and the like, particularly where the devices or lenses are intended to be used in contact with a patient or wearer. For applications of this type, the compositions may be conveniently provided in the form of a liquid or foam, and may be provided with emulsifiers, surfactants, buffering agents, wetting agents, preservatives, and other components commonly found in compositions of this type. Compositions of the invention may be impregnated into absorptive materials, such as sutures, bandages, and gauze, or coated onto the surface of solid phase materials, such as staples, zippers and catheters, which are packaged and maintained under substantially anaerobic conditions, such as in sealed foil and/or polymer pouches or bags that are impervious to air. The pouches or bags may then be opened to deliver the compositions to a site for the prevention of microbial infection. Other delivery systems of this type will be readily apparent to those skilled in the art.

Actual amounts of haloperoxidase, halide, peroxide generating agent and/or antimicrobial activity enhancing agents in the compositions of the invention may be varied so as to obtain amounts of haloperoxidase and antimicrobial activity enhancing agents at the site of treatment effective to kill vegetative microorganisms as well as yeast and sporular microorganisms. Accordingly, the selected amounts will depend on the nature and site for treatment, the desired response, the desired duration of microbicidal action and other factors. Generally, when the haloperoxidase is myeloperoxidase, eosinophil peroxidase, lactoperoxidase or compositions thereof, liquid compositions of the invention will comprise at least 0.01 picomoles (pmol) of haloperoxidase per ml of liquid composition, more preferably from about 0.1 pmol to about 500 pmol of haloperoxidase per ml of liquid composition, and most preferably from about 0.5 pmol to about 50 pmol of myeloperoxidase per ml of liquid composition. Optionally, it may be desirable in some applications to include both eosinophil peroxidase and myeloperoxidase in the same composition. Liquid compositions of the invention will generally further comprise from 100 μ mol/ml to 300 μ mol/ml chloride, from 10 μ mol to 50 μ mol/ml bromide, from 1 μ mol/ml to 5 μ mol/ml iodide, or combinations thereof. Optionally, liquid compositions of the invention may generally comprise at least 0.005 μ mol/ml of antimicrobial activity enhancing agents, i.e., α -amino acids such as glycine and alanine, and more preferably from 0.05 μ mol/ml to 50 μ mol/ml of such antimicrobial activity enhancing agent.

Finally, liquid compositions of the invention may generally comprise from 0.05 to 10 units/ml of an enzyme, such as glucose oxidase, capable of oxidizing a substrate, such as glucose, and reducing oxygen to hydroge peroxide; and may additionally comprise from 0.1 to 10 $\mu\text{mol/ml}$ of a substrate for the enzyme. The foregoing

5 components will typically be combined in a pharmaceutically acceptable liquid carrier.

As an illustrative example, a composition suitable for use as a contact lens solution may comprise from 1 to 20 $\mu\text{mol/ml}$ of eosinophil peroxidase and/or myeloperoxidase, from 0.1 to 10 $\mu\text{mol/ml}$ of glycine, from 0.01 to 10 units of glucose oxidase, and from 50 to 300 mEq/L of chloride with 0.1 to 5 mEq/L bromide. The
10 above composition is combined with from 0.1 to 10 $\mu\text{mol/ml}$ of glucose under anaerobic conditions and the complete preparation is kept anaerobic until used as a liquid disinfectant or sterilizing solution. Exposure to air, i.e., molecular oxygen, activates the disinfecting-sterilizing action of the formulation.

Due to the selective binding capacities of myeloperoxidase and eosinophil
15 peroxidase, even relatively high potency formulations are of low toxicity to mammalian tissue. Furthermore, the ability to temporally limit the period of maximum potency further decreases the potential for host toxicity by confining the high potency disinfection-sterilization period to the time prior to host contact. As such, a material or device treated with the disinfectant-sterilant can be brought in direct contact with
20 host tissue in the post sterilization period.

These and other aspects of the invention may be better understood in connection with the following examples, which are provided for purposes of illustration and not by way of limitation.

Example 1

25 Anaerobic Formulation with Microbicidal Activity on Air Exposure

A gas tight glove box was prepared as an anaerobic chamber by first gassing with nitrogen (N_2 , 99.99% O_2 -free). After the percentage of O_2 had fallen to 0.1%, 5 anaerobic gas packs were opened and activated in the chamber. The following solutions and bacterial suspension were prepared and placed in the anaerobic
30 chamber:

(a₁) Glucose Oxidase (GOX) prepared from Type VII *Aspergillus niger* GOX (Sigma Chemicals).

(a₂) Acetate Buffer without GOX.

(b) Myeloperoxidase (MPO, Lot #1899201, ExOxEmis, Inc) solution
35 containing D-glucose, chloride and a trace bromide.

(c) Suspension of *Staphylococcus aureus*.

Once the O₂ concentration had stabilized at between 0.01 and 0.02%, i.e., 100 to 200 parts per million (ppm), solution (a₁) or solution (a₂) was mixed with (b), and a portion of each mixture and a portion of the bacterial suspension (c) were removed from the chamber. After approximately ten minutes, the bacterial suspension was added to the aerobic and anaerobic mixtures with GOX (a₁) and without GOX (a₂). The final solutions contained either 0.6 units GOX or no GOX, 56 micromoles (μmol) D-glucose, 5 picomoles (pmol) of MPO, and approximately 3 x 10⁷ *Staphylococcus aureus* bacteria per ml of 50 mM acetate buffer with 100 mEq/l Cl⁻ and 1 mEq/l Br⁻, pH 6, in the presence and in the absence of air (ambient O₂). Samples (100 μl of mixed suspension) were removed from the test solutions at 1.25, 2.5, 5, 10 and 20 min and immediately diluted with 0.9 ml of 0.1% thioglycollate containing 200 units catalase (Sigma Chemicals) to terminate oxidative killing. The samples were further diluted and plated on trypticase soy agar (hockey stick technique). The bacterial colonies were counted after 1 to 2 days of incubation at 37°C, and *Staphylococcus aureus* survival is expressed as colony forming units (CFU) per ml of the original suspension as shown in Table 1. As used in Table 1 and the following examples, 0 indicates no growth at the lowest dilution tested; i.e., less than 100 CFU.

Table 1
Oxygen-Dependent Killing of *Staphylococcus aureus*

Time in Min	<i>Staphylococcus aureus</i> (CFU/ml)			
	GOX (None) + MPO (5 pmol)		GOX (0.6 unit) + MPO (5 pmol)	
	Anaerobic	Aerobic	Anaerobic	Aerobic
1.25	31,000,000	28,400,000	24,400,000	0
2.5	25,200,000	26,200,000	24,800,000	0
5	29,600,000	26,000,000	25,600,000	0
10	26,000,000	39,400,000	900,000	0
20	22,000,000	27,400,000	760,000	0

Complete killing of *Staphylococcus aureus* was observed for the GOX-MPO complete system in the presence of O₂. Although no killing was observed from the GOX-MPO complete system in the absence of O₂ during the initial 5 min interval, incomplete killing was observed after 10 and 20 min exposure. This incomplete killing can be explained by the fact that the anaerobic chamber still contained 0.01 to 0.02% O₂. Although the O₂ concentration in the anaerobic chamber is about one-thousandth that of air, this quantity of O₂ is sufficient to produce a partial

microbicidal action. No killing was observed with the MPO-only system (no GOX) in the presence or absence of O₂.

Example 2

Anaerobic Preparation and O₂-Activation of Microbicidal-Sporicidal Activity of High Potency MPO- and EPO-Based Formulations

5

The anaerobic chamber was prepared as described in Example 1, but the components comprising the GOX-XPO formulation were changed and glycine was added to facilitate sporicidal action, as described in Allen, U.S. Patent No. 5,389,369. Two different formulations were anaerobically prepared. The first formulation
10 contained 0.5 units GOX (Type VII *Aspergillus niger* GOX, Sigma Chemicals) plus 56 µmol D-glucose, 30 pmol MPO (porcine, Lot #1899201, ExOxEmis, Inc.) and 2 µmol glycine per ml of 50 mM acetate buffer with 100 mEq/l Cl⁻ and 1 mEq/l Br⁻, pH 6. The second formulation was the same but with 30 pmol EPO (Lot # 1929201, ExOxEmis, Inc.) substituted for MPO. Both formulations were then allowed to age
15 anaerobically for over a week before testing.

15

The microbicidal capacity of the GOX-MPO-glycine formulation was tested after 12 days of anaerobic storage. As indicated in Table 2, the formulation was tested against a broad range of gram negative and gram positive bacteria, yeast and fungal spores. Each test condition contained 400 µl of the GOX-MPO-glycine
20 formulation and 200 µl of microbial suspension, and was activated by exposure to air (ambient O₂). Samples were removed after 0, 5, 10, and 20 min incubation (for the bacteria) and after 20, 30, 60 and 90 min incubation (for the yeast and fungal spores), and immediately diluted with 0.9 ml of 0.1% thioglycollate containing 200 units of catalase, diluted, and plated on trypticase soy agar (bacteria) or Sabouraud's dextrose
25 agar (yeast and fungi). The colonies were counted after 1 to 4 days of incubation at 37°C.

25

The MPO concentration (18 pmol/ml, final) of this formulation effectively killed all gram negative and gram positive bacteria tested including Group A streptococcus. Killing was complete after 5 to 20 min exposure. The formulation
30 also effectively killed the yeast and fungal spores tested, but a longer exposure, i.e., 30 to 90 min, was required for complete killing.

30

Table 2
Anaerobic Versus Aerobic Microbicidal Action of
GOX-MPO-Glycine Formulation

0.3 units GOX, 18 μ mol MPO & 1 μ mol glycine (final reaction conc. per ml)			
Time in Min	<i>Escherichia coli</i>	<i>Serratia marcesans</i> ATCC 14041	<i>Pseudomonas</i> <i>aeruginosa</i>
0	45,400,000	21,000,000	26,000,000
5	0	13,800,000	4,200,000
10	0	50,000	6,000
20	0	0	0

Time in Min	<i>Staphylococcus aureus</i>	<i>Streptococcus pyogenes</i> (Group A)
0	21,600,000	2,880,000
5	60,000	0
10	6,000	0
20	0	0

Time in Min	<i>Candida albicans</i>	<i>Aspergillus fumigatus</i>	<i>Fusarium moniliforme</i>
0	1,100,000	100,000	88,000
20	420,000	280,000	54,000
30	166,000	0	4,400
60	0	0	800
90	0	0	0

The microbicidal capacity of the GOX-EPO-glycine formulation was tested after 13 days of anaerobic storage. This EPO-based formulation was tested against the same microbes under the same test conditions. The results are shown in Table 3.

This EPO-based formulation was as effective as the previously considered MPO-based formulation and was quicker acting. At this EPO concentration (18 μ mol/ml, final) all gram negative and gram positive bacteria, including Group A streptococcus, were completely killed after 5 min exposure. Killing of *Candida albicans* and *Fusarium moniliforme* spores was also complete by 90 min.

Table 3
Anaerobic Versus Aerobic Microbicidal Action of GOX-EPO-Glycine
Formulation

0.3 units GOX, 18 μ mol EPO & 1 μ mol glycine (final reaction conc. per ml)			
Time in Min	<i>Escherichia coli</i>	<i>Serratia marcesans</i> ATCC 14041	<i>Pseudomonas</i> <i>aeruginosa</i>
0	47,400,000	34,800,000	33,200,000
5	0	0	0
10	0	0	0
20	0	0	0

Time in Min	<i>Staphylococcus aureus</i>	<i>Streptococcus pyogenes</i> (Group A)
0	19,200,000	1,000,000
5	0	0
10	0	0
20	0	0

Time in Min	<i>Candida albicans</i>	<i>Fusarium moniliforme</i>
0	740,000	116,000
20	10,160	7,600
30	9,600	4,200
60	0	800
90	0	0

5

Example 3

Preparation and Testing of O₂-Activated Microbicidal-Sporicidal Formulations Packaged Pressurized Spray Cans

Preparation of Spray Sterilent:

- 10 *Preparation of Stock Solutions:* A 10% (volume/volume) Tween 80 solution was prepared by adding 10 ml Tween 80 to 90 ml H₂O. A 1% (weight/volume) EDTA solution was prepared by adding 1 g Na₂EDTA to 100 ml H₂O. A 0.1 M glycine solution was prepared by adding 1.5 g glycine (M.W. 75) to 200 ml H₂O. A 0.5% (w/v) hydroxypropyl-methyl-cellulose (HPMC, 100 centipoise) solution was
- 15 prepared by adding 1 g HPMC to 200 ml H₂O and gently mixing until fully dissolved. A 250 unit/ml GOX (Type VII from *Aspergillus niger*, Sigma Chemicals) was prepared in H₂O. A 0.1 M glucose solution was prepared by adding 1.8 g D-glucose (M.W. 180) to 100 ml H₂O.

Preparation of Simple Sterilent Working Stock:

	Weight/Volume	Stock Solution	Final Concentration
(1.)	5 ml	10% Tween 80	0.1%
(2.)	50 ml	1% EDTA	0.1%
(3.)	10 ml	0.1 M Glycine	2 mM
(4.)	4 ml	250 units/ml GOX	2 units/ml
(5.)	0.73 g	NaCl	25 mM
(6.)	0.05 g	NaBr	1 mM
(7.)	1.79 ml	MPO _{porcine}	50 pmol/ml
(8.)	quantum sufficit (qs) for 500 ml with Acetate Buffer, pH 5.5 (10 mM final)		

The ingredients described above were added to 300 ml H₂O in the order indicated. Each stock addition was completely dissolved before the next addition. The working stock and D-glucose solutions were sterilized by passage through a 0.22 micron filter, and both solutions were placed in the anaerobic chamber. Once the chamber had stabilized at approximately 20 to 100 ppm O₂, 40 ml 0.1 M D-glucose were added to the working stock for a final concentration of 7 mM (130 mg/dL).

Approximately 100 ml of the complete *Simple Sterilent Solution* were added to 45 x 165 mm EP Spray System canisters (full capacity 140 ml) through a flap valve using an adapter-fitted syringe. The cans were then removed from the chamber and the outer compartment of the canister was pressurized with nitrogen gas (N₂).

Preparation of Complex Working Stock:

	Weight/Volume	Stock Solution	Final Concentration
(1.)	5 ml	10% Tween 80	0.1%
(2.)	50 ml	1% EDTA	0.1%
(3.)	50 ml	0.5% HPMC	0.05%
(4.)	10 ml	0.1 M Glycine	2 mM
(5.)	4 ml	250 units/ml GOX	2 units/ml
(6.)	2.92 g	NaCl	100 mM
(7.)	0.05 g	NaBr	1 mM
(8.)	1.79 ml	MPO _{porcine}	50 pmol/ml
(9.)	qs for 500 ml with Acetate Buffer, pH 6 (10 mM final).		

The ingredients described above were added to 300 ml H₂O in the order indicated. Each stock addition was completely dissolved before the next addition. The working stock and D-glucose solutions were sterilized by passage through a 0.22 micron filter, and both solutions were placed in the anaerobic chamber. Once the chamber had stabilized at approximately 20 to 30 ppm O₂, 40 ml 0.1 M D-glucose were added to the working stock for a final concentration of 7 mM (130 mg/dL). Approximately 100 ml of the complete *Complex Sterilent Solution* were added to

each EP Spray System canister, and the canisters were pressurized with N₂ as previously described.

- To test the microbicidal capacities of the *Simple Sterilent Solution* and the *Complex Sterilent Solution* against *Escherichia coli* and *Aspergillus fumigatus* (spores), 0.4 ml aliquots of freshly sprayed *Simple Sterilent Solution* (4 canisters tested) and *Complex Sterilent Solution* (3 canisters tested) were added to 0.1 ml of the microbe suspensions in the presence of air. *A. fumigatus* plates were read after 120 hrs incubation. The canister preparations were 16 days old at the time of testing. The results are presented in Table 4. Both sterilent solutions produced potent bactericidal action. Fungal sporicidal action was present but incomplete at 90 min exposure.

Table 4
Microbicidal Activity of the *Simple* and *Complex Sterilent Solutions*
on Air Exposure

Sterilent Solution Tested	<i>Escherichia coli</i> CFU/ml after 30 min	<i>Aspergillus fumigatus</i> CFU/ml after 90 min
Control	175,600,000	940,000
Simple	0	46,000
Simple	0	32,000
Simple	0	60,000
Simple	0	40,000
Complex	0	800
Complex	0	56,000
Complex	0	44,000

- The shelf life of the canisters of sterilent solution was tested by aging the preparations at 4°C, room temperature and 40°C for approximately 1 month, and then repeating the procedure described above in connection with Table 4. The results are shown in Table 5.

Table 5
Effect of Temperature on the Microbicidal Capacity of the Simple
and Complex Sterilent Solution Canisters

Sterilent Solution Tested	<i>Escherichia coli</i> CFU/ml after 30 min	<i>Aspergillus fumigatus</i> CFU/ml after 90 min
Control	48,000,000	1,020,000
Simple		
4°C	0	220,000
23°C	0	220,000
40°C	0	180,000
Complex		
4°C	0	260,000
23°C	0	220,000
40°C	0	200,000

As shown in Table 5, both the Simple Sterilent Solution and the Complex Sterilent Solution maintained full potency against *E. coli* after storage for 1 month. Fungicidal activity after the storage period against *A. fumigatus* was also observed.

Example 4

Preparation, luminescence-based quality control, and microbicidal capacity of a high potency O₂-activated microbicidal-sporicidal formulation.

10 Preparation of High Potency Working Stock:

	Weight/Volume	Stock Solution	Final Concentration
(1.)	100 ml	10% Tween 80	0.1%
(2.)	1,000 ml	1% EDTA	0.1%
(3.)	1,000 ml	0.5% HPMC	0.05%
(4.)	200 ml	0.1 M Glycine	2 mM
(5.)	200 ml	250 units/ml GOX	5 units/ml
(6.)	59.2 g	NaCl	100 mM
(7.)	1.0 g	NaBr	1 mM
(8.)	107.6 ml	MPO _{porcine}	150 pmol/ml
(9.)	qs for 10 liters with Acetate Buffer, pH 6.5 (10 mM final)		

The ingredients described above were mixed as described in Example 3. The working stock and a 0.56 M D-glucose solution were sterilized by passage through a 0.22 micron filter, and both solutions were placed in the anaerobic chamber. The large scale of this production made lowering and maintaining the O₂ concentration difficult. The minimum O₂ concentration achieved was 35 ppm. 125 ml 0.56 M D-glucose were added to the working stock for a final glucose concentration of

6.9 mM (130 mg/dL). Approximately 100 ml of the complete *High Potency Sterilent Solution* were added to each EP Spray System canister, and the canisters were pressurized with N₂ as described in Example 3. Ninety-nine canisters were filled.

The microbicidal capacities of early, middle and late production canisters were tested using the methodology previously described for the Table 4 data. *Escherichia coli* (119,800,000 CFU/test) were completely killed within 30 min by all of the canisters of sterilent tested. Although killing of *Aspergillus fumigatus* spores (2,300,000 CFU/test) was incomplete, the canisters of sterilent produced a hundredfold kill after 90 min exposure at room temperature.

10

Example 5

Preparation and Testing of O₂-Activated Disinfectant-Sterilent Solutions Using Different Substrate-Oxidase Drivers

Preparation of Lens Disinfectant-Sterilent Solution:

Preparation of Stock Solutions: Tween 80 and Na₂EDTA solutions were prepared as described in Example 3. A 1.0 M glycine solution was prepared by adding 75 g glycine (M.W. 75) to 1 liter H₂O. A 1.0% (w/v) hydroxypropyl-methylcellulose (HPMC, 100 centipoise) solution was prepared by adding 5 g HPMC to 500 ml H₂O and gently mixing until fully dissolved. Acetate Buffer (20 mM, pH 6.8) was prepared by adding 1.2 ml glacial acetic acid (C₂H₄O₂, M.W. 60) and 1.64 g of sodium acetate (NaC₂H₃O₂, M.W. 82) per liter of H₂O.

20

Preparation of Common Working Stock:

	Weight/Volume	Stock Solution	Final Concentration
(1.)	0.2 liters	10% Tween 80	0.1%
(2.)	1.0 liters	1% EDTA	0.05%
(3.)	2.0 liters	1% HPMC	0.1%
(4.)	50 ml	1.0 M Glycine	2.5 mM
(5.)	171 g	NaCl	145 mM
(6.)	2.0 g	NaBr	1 mM
(7.)	qs for 20 liters with Acetate Buffer (10 mM final). Adjust pH to 6.8 with HCl/NaOH as required prior to filtration. Estimated osmolality 310 mOsm; range of osmolality for tears is 309 to 347 mOsm.		

The ingredients were mixed as previously described. The working stock was used to prepare four different substrate-oxidase preparations. The four oxidases tested were: (1) choline oxidase, (2) glycerol-3-phosphate oxidase, (3) galactose oxidase, and (4) D-amino acid oxidase. The final oxidase activity of all of the preparations was 1 unit/ml of the final preparation. Choline, glycerol-3-phosphate, D-galactose and glycine were included at a final concentration of 2.5 mM as substrates

25

for the oxidases. The MPO concentration of the preparation was approximately 100 pmol/ml. The solutions were sterilized by passage through a 0.22 micron filter and placed in the anaerobic chamber. The minimum O₂ concentration achieved was 10 ppm. After depletion of O₂, the oxidases and their specific substrates were added to the various preparations in the anaerobic chamber. Approximately 100 ml of each of the complete *Substrate-Oxidase Sterilent Solutions* were added to each EP Spray System canister, and the canisters were pressurized with N₂ as described in Example 3.

These experiments were designed to test the possibilities of using substrate-oxidase combinations other than glucose-glucose oxidase to formulate O₂-activated disinfectant-sterilent solutions. Using the procedure of Example 3, the antimicrobial activity of each preparation was tested against *E. coli* and *S. aureus*. The results of testing are presented in Table 6, in which "Dilution" indicates the dilution of the sterilent; i.e., the final ratio of MPO:Oxidase to microbe suspension, and 0 indicates no growth at the lowest dilution tested; i.e., less than 100 CFU.

Table 6
Microbicidal Capacities of Various Substrate-Oxidase Formulations

Formulations, MPO:Oxidase Combinations: Dilution 1:1.1	<i>Escherichia coli</i> CFU/ml after 30 min	<i>Staphylococcus aureus</i> CFU/ml after 30 min
Control	6,400,000	9,400,000
MPO:Choline Ox (1U)	34,000	8,800,000
MPO:Glycerol-3-P Ox (1U)	110,000	1,110,000
MPO:Galactose Ox (1U)	0	0
MPO:D-AA Ox (1U)	22,000	0

Each of the substrate-oxidase formulations of Table 6 showed microbicidal action, but none of the preparations tested demonstrated any special advantage over the previously tested glucose-glucose oxidase system.

Example 7 **Preparation and Testing of O₂-Activated Disinfectant-Sterilent Formulations for Ophthalmic Use**

Preparation of Lens Disinfectant-Sterilent Solution:

Preparation of Stock Solutions: The stock solutions were essentially the same as described for Example 6.

Preparation of Lens Disinfectant-Sterilent Working Stock:

	Weight/Volume	Stock Solution	Final Concentration
(1.)	10 ml	10% Tween 80	0.1%
(2.)	50 ml	1% EDTA	0.05%
(3.)	100 ml	1% HPMC	0.1%
(4.)	30 ml	0.1 M Glycine	3.0 mM
(5.)	8.76 g	NaCl	150 mM
(6.)	0.1 g	NaBr	1 mM
(7.)	30 ml	0.1 M D(+)-Glucose	3 mM
(8.)	qs for 1 liter with 20 mM Acetate Buffer. Adjust final pH to 6.8 with HCl/NaOH as required prior to filtration. Estimated osmolality 325mOsm; the osmolality for tears is 309 to 347 mOsm.		

The ingredients were mixed as previously described to produce the working stock. The glucose oxidase Type VII (1,000 units GOX/ml) was added to the stock to prepare four formulations:

5 *Formulation A:* 0.5 ml GOX/liter solution \therefore 0.5 units/ml (final).

Formulation B: 1 ml GOX/liter solution \therefore 1 units/ml (final).

Formulation C: 2 ml GOX/liter solution \therefore 2 units/ml (final).

Formulation D: 4 ml GOX/liter solution \therefore 4 units/ml (final).

The MPO concentration of the preparation (undiluted) was approximately
 10 100 pmol/ml. The solutions were sterilized by passage through a 0.22 micron filter and placed in the anaerobic chamber. The minimum O₂ concentration achieved was 10 ppm. After depletion of O₂, the oxidases and their specific substrates were added to the various preparations in the anaerobic chamber. Approximately 100 ml of each of the complete *Lens Disinfectant-Sterilent Formulations* were added to each EP
 15 Spray System canister, and the canisters were pressurized with N₂ as previously described.

These four preparations were designed and tested to achieve a contact lens care disinfectant-sterilent formulation with excellent microbicidal-sporicidal capacity and minimum potential for host toxicity. The experiments with these formulations
 20 were also designed to answer questions regarding duration of activity after exposure to O₂ and long range shelf stability of the canister preparations. The microbicidal-sporicidal activities of the formulations immediately, 2 hours and 4 hours after air exposure against *S. aureus*, *E. coli* and *A. fumigatus* were tested using the procedure of Example 3. The results are shown in Table 7. The formulations had been
 25 packaged in the canisters and stored for six months at the time of testing.

Table 7
Microbicidal Activity Relative to Period of Air Exposure for Ophthalmic Disinfectant-Sterilent Formulations (6 Months After Manufacture)

Formulation Tested	Time, Post O ₂ Exposure	CFU/ml after 1 Hour		
		<i>Staph. aureus</i>	<i>E. coli</i>	<i>A. fumigatus</i>
Control	Immediate	285,200,000	221,600,000	5,500,000
Control-MPO	Immediate	263,000,000	101,600,000	5,360,000
<i>Formulation A</i>	Immediate	0	0	4,340,000
<i>Formulation B</i>	Immediate	0	0	760,000
<i>Formulation C</i>	Immediate	0	0	5,200
<i>Formulation D</i>	Immediate	0	0	400
<i>Formulation A</i>	2 Hours	0	0	420,000
<i>Formulation B</i>	2 Hours	0	0	5,800
<i>Formulation C</i>	2 Hours	0	0	600
<i>Formulation D</i>	2 Hours	0	0	200
<i>Formulation A</i>	4 Hours	0	0	64,000
<i>Formulation B</i>	4 Hours	0	0	6,200
<i>Formulation C</i>	4 Hours	0	0	0
<i>Formulation D</i>	4 Hours	0	0	0

- Complete *Staphylococcus aureus* and *Escherichia coli* killing was observed for all of the formulations at all of the post-O₂ exposure times tested. Killing of *Aspergillus fumigatus* spores immediately after exposing the formulations to air was proportional to the GOX concentration of the formulations. *Formulation A* (0.5 units GOX/ml) produced only minimal killing. *Formulations B, C and D* with progressively higher GOX activities produced progressively greater killing. The formulations showed even better microbicidal-sporicidal activity at 2 hours and 4 hours post-O₂ exposure. In fact, the formulations were most effective at 4 hours post-O₂ exposure. In order to further investigate this trend, the experiment was extended to include longer post-O₂ exposure times, using formulations that had been stored in canisters for 7 months. The results of testing are presented in Table 8.

Table 8
Microbicidal Activity Relative to Period of Air Exposure for the *Ophthalmic Disinfectant-Sterilent Formulations* (7 Months After Manufacture)

Formulation Tested	Time, Post O ₂ Exposure	CFU/ml after 1 Hour.		
		<i>Staph. aureus</i>	<i>E. coli</i>	<i>A. fumigatus</i>
Control	4 Hours	97,000,000	323,200,000	6,420,000
Control-MPO	4 Hours	81,600,000	296,800,000	3,160,000
<i>Formulation A</i>	4 Hours	0	0	1,400,000
<i>Formulation B</i>	4 Hours	0	0	1,200,000
<i>Formulation C</i>	4 Hours	0	0	100,000
<i>Formulation D</i>	4 Hours	0	0	1,600
<i>Formulation A</i>	8 Hours	0	0	720,000
<i>Formulation B</i>	8 Hours	0	0	600
<i>Formulation C</i>	8 Hours	0	0	600
<i>Formulation D</i>	8 Hours	0	0	0
<i>Formulation A</i>	12 Hours	5,000	0	340,000
<i>Formulation B</i>	12 Hours	0	0	27,000
<i>Formulation C</i>	12 Hours	0	0	4,200
<i>Formulation D</i>	12 Hours	0	0	2,200
<i>Formulation A</i>	24 Hours	206,000	0	1,860,000
<i>Formulation B</i>	24 Hours	0	0	98,000
<i>Formulation C</i>	24 Hours	0	0	184,000
<i>Formulation D</i>	24 Hours	0	0	9,400

The results for the 4-hour post-O₂ exposure testing are essentially in agreement for both the initial (Table 7) and follow-up experiment (Table 8). The microbicidal-sporicidal activities of the formulations at 8-hour post-O₂ exposure remain the same or slightly increased. However, at 12 hours and especially at 24 hours post-O₂ exposure, a small decrease in *Staphylococcus aureus* and *Aspergillus fumigatus* spore killing was noted.

As disclosed in U.S. Patent No. 5,389,369, fungal spore killing requires a relatively long time period of exposure to MPO:GOX disinfectant-sterilent solutions. As such, the 12- and 24-hours post-O₂ exposure experiments of Table-8 were repeated, using formulations that had been stored in canisters for 8 months, to allow comparison of bactericidal-fungicidal action resulting from 1 hour and 4 hours exposure to the disinfectant-sterilent formulations. The results are presented in Table 9.

Table 9
Microbicidal Activity Relative to Period of Air Exposure and
Period of Microbial Contact for the *Ophthalmic Disinfectant-Sterilent*
Formulations (8 Months After Manufacture)

Formulation Tested	Time, Post O ₂ Exposure	CFU/ml		
		<i>Staph. aureus</i>	<i>E. coli</i>	<i>A. fumigatus</i>
Incubation Time, 1 Hour				
Control	12 Hours	169,600,000	142,800,000	3,560,000
Control-MPO	12 Hours	145,600,000	140,600,000	3,380,000
<i>Formulation A</i>	12 Hours	4,200	0	1,200,000
<i>Formulation B</i>	12 Hours	660,000	0	13,800
<i>Formulation C</i>	12 Hours	0	0	3,600
<i>Formulation D</i>	12 Hours	40,000	0	2,200
Incubation Time, 4 Hours				
<i>Formulation A</i>	12 Hours	0	0	0
<i>Formulation B</i>	12 Hours	0	0	0
<i>Formulation C</i>	12 Hours	0	0	0
<i>Formulation D</i>	12 Hours	0	0	0
<i>Formulation A</i>	24 Hours	0	0	0
<i>Formulation B</i>	24 Hours	0	0	0
<i>Formulation C</i>	24 Hours	0	0	0
<i>Formulation D</i>	24 Hours	0	0	0

- 5 The microbicidal capacities of the formulations, after storage in the canisters for 11 months, were tested against *Pseudomonas aeruginosa* and *Candida albicans* following post-O₂ exposure periods up to 48 hours. Kill capacity was measured for both 1 hour and 4 hours exposure periods. The results are presented in Table 10.

Table 10
Microbicidal Activity Relative to Period of Air Exposure and Period of
Microbial Contact for the *Lens Disinfectant-Sterilent*
Formulations (10 Months After Manufacture)

Formulation Tested	Time, Post O ₂ Exposure	<i>Pseudomonas aeruginosa</i>		<i>Candida albicans</i>	
		1 Hour	4 Hours	1 Hour	4 Hours
Control	Immediate	400,000,000	315,200,000	5,620,000	7,020,000
Control-MPO	Immediate	344,000,000	313,600,000	5,860,000	7,820,000
<i>Formulation A</i>	Immediate	0	0	7,120,000	2,760,000
<i>Formulation B</i>	Immediate	0	0	2,260,000	620,000
<i>Formulation C</i>	Immediate	0	0	3,000	0
<i>Formulation D</i>	Immediate	0	0	0	0
Control	12 Hours	314,400,000	306,400,000	4,920,000	2,500,000
Control-MPO	12 Hours	320,000,000	264,000,000	5,100,000	2,800,000
<i>Formulation A</i>	12 Hours	600	0	132,000	1,000
<i>Formulation B</i>	12 Hours	600	0	2,000	0
<i>Formulation C</i>	12 Hours	0	0	0	0
<i>Formulation D</i>	12 Hours	0	0	0	0
Control	24 Hours	276,800,000	275,200,000	5,000,000	2,360,000
Control-MPO	24 Hours	204,800,000	249,600,000	5,180,000	4,540,000
<i>Formulation A</i>	24 Hours	0	0	0	0
<i>Formulation B</i>	24 Hours	0	0	0	0
<i>Formulation C</i>	24 Hours	0	0	0	0
<i>Formulation D</i>	24 Hours	0	0	0	0
Control	48 Hours	336,000,000	252,000,000	1,000,000	3,240,000
Control-MPO	48 Hours	236,800,000	200,800,000	3,620,000	3,820,000
<i>Formulation A</i>	48 Hours	0	0	0	0
<i>Formulation B</i>	48 Hours	0	0	0	0
<i>Formulation C</i>	48 Hours	0	0	0	0
<i>Formulation D</i>	48 Hours	0	0	0	0

5 Eleven months after manufacture, all of the formulations produced total kill of *Pseudomonas aeruginosa* and *Candida albicans* at 24 hours post-O₂ exposure times and longer.

Finally, after canister storage for a period of 1 year, the microbicidal activity against *E. coli*, *P. aeruginosa*, *C. albicans* and *A. fumigatus* of various dilutions of

Formulation D (described above) relative to air exposure times up to 24 hours was determined. The results are shown in Table 11.

Table 11
Microbicidal Activity of Various Dilutions of Formulation D Relative to
Air Exposure Time for the *Lens Disinfectant-Sterilent Formulations*
(1 Year After Manufacture)

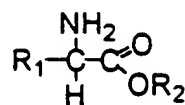
Formulation D Dilution Tested	Time Post O ₂ Exposure	MPO		GOX		CFU's after 2 Hours Exposure			
		pmol/ml	µg/ml	Units/ml		<i>E. Coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>Asp. fumigatus</i>
Control	Immediate	0	0	0		280,000,000	81,600,000	3,340,000	2,560,000
Neat	Immediate	50	7	2		0	0	0	0
1:2 Dil.	Immediate	25	4	1		0	0	1,440,000	0
1:4 Dil.	Immediate	12	2	0.5		0	0	3,280,000	840,000
1:8 Dil.	Immediate	6	1	0.25		99,200,000	0	1,940,000	2,260,000
1:16 Dil.	Immediate	3	0.5	0.12		235,200,000	90,400,000	2,540,000	2,460,000
Control	12 Hours	0	0	0		212,800,000	57,600,000	2,520,000	5,560,000
Neat	12 Hours	50	7	2		0	0	0	0
1:2 Dil.	12 Hours	25	4	1		0	0	3,200	1,600
1:4 Dil.	12 Hours	12	2	0.5		0	0	3,000,000	2,480,000
1:8 Dil.	12 Hours	6	1	0.25		55,200,000	0	980,000	2,920,000
1:16 Dil.	12 Hours	3	0.5	0.12		113,600,000	26,400,000	2,980,000	3,240,000
Control	24 Hours	0	0	0		320,000,000	63,200,000	4,080,000	3,600,000
Neat	24 Hours	50	7	2		0	0	0	0
1:2 Dil.	24 Hours	25	4	1		0	0	0	0
1:4 Dil.	24 Hours	12	2	0.5		12,000	0	2,680,000	2,400
1:8 Dil.	24 Hours	6	1	0.25		18,000,000	0	2,500,000	1,720,000
1:16 Dil.	24 Hours	3	0.5	0.12		145,600,000	17,600	2,320,000	1,780,000

As shown in Table 11, after a full year of storage, Formulation D remained highly active against all organisms tested.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without
5 departing from the spirit and scope of the invention.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for killing or inhibiting the growth of microorganisms comprising the steps of:
 - (a) maintaining under substantially anaerobic conditions a microbicidal composition comprising a haloperoxidase, a halide, and a peroxide generating agent capable of generating peroxide upon exposure to oxygen;
 - (b) exposing the composition to oxygen to activate the microbicidal activity of the composition; and
 - (c) contacting the microorganisms with the activated composition to kill or inhibit the growth of the microorganisms.
2. The method of Claim 1 wherein the haloperoxidase is selected from the group consisting of myeloperoxidase, eosinophil peroxidase and lactoperoxidase.
3. The method of Claim 1 wherein the halide is selected from the group consisting of chloride, bromide, iodide and combinations thereof.
4. The method in Claim 1 wherein the peroxide generating agent is an enzyme capable of oxidizing a substrate and reducing oxygen to hydrogen peroxide.
5. The method of Claim 4 wherein the enzyme is glucose oxidase and the substrate is glucose.
6. The method of Claim 1 wherein the haloperoxidase is myeloperoxidase.
7. The method of Claim 1 wherein the haloperoxidase is eosinophil peroxidase and the halide is selected from the group consisting of bromide, iodide and combinations thereof.
8. The method of Claim 1 wherein the haloperoxidase is lactoperoxidase and the halide is selected from the group consisting of bromide, iodide and combinations thereof.
9. The method of Claim 1 wherein the microbicidal composition further comprises an antimicrobial activity enhancing agent of the formula:



wherein R_1 is hydrogen, an unsubstituted, or hydroxy or amino substituted, straight or branched chain alkyl group having from 1 to 6 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbons.

10. The method of Claim 9 wherein the antimicrobial activity enhancing agent is an α -amino acid selected from the group consisting of glycine and the *L*- or *D*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine and the alkyl esters thereof.

11. The method of Claim 1 wherein the microbicidal composition is maintained under anaerobic conditions by packaging the microbicidal composition under pressure in a hermetically sealed container for dispensing as a liquid, a foam or a gel.

12. The method of Claim 1 wherein the microbicidal composition is maintained under anaerobic conditions by impregnating the microbicidal composition in a tangible substrate, and then hermetically sealing the impregnated tangible substrate in a closed container.

13. A hermetically sealed container comprising, under substantially anaerobic conditions, an antimicrobial formulation comprising a haloperoxidase, a halide, and a peroxide generating agent capable of generating peroxide upon exposure to oxygen, and means for releasing the formulation from the container.

14. The container of Claim 13 wherein the haloperoxidase is selected from the group consisting of myeloperoxidase, eosinophil peroxidase and lactoperoxidase.

15. The container of Claim 13 wherein the halide is selected from the group consisting of chloride, bromide, iodide and combinations thereof.

16. The container of Claim 13 wherein the peroxide generating agent is an enzyme capable of oxidizing a substrate and reducing oxygen to hydrogen peroxide.

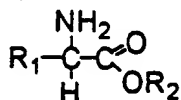
17. The container of Claim 16 wherein the enzyme is glucose oxidase and the substrate is glucose.

18. The container of Claim 13 wherein the haloperoxidase is myeloperoxidase.

19. The container of Claim 13 wherein the haloperoxidase is eosinophil peroxidase and the halide is selected from the group consisting of bromide, iodide and combinations thereof.

20. The container of Claim 13 wherein the haloperoxidase is lactoperoxidase and the halide is selected from the group consisting of bromide, iodide and combinations thereof.

21. The composition of Claim 10 wherein the antimicrobial activity enhancing agent of the formula:



wherein R₁ is hydrogen, an unsubstituted, or hydroxy or amino substituted, straight or branched chain alkyl group having from 1 to 6 carbon atoms, and R₂ is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbons.

22. The container of Claim 18 which comprises at least 0.01 pmol/ml of myeloperoxidase in a liquid carrier.

23. The container of Claim 18 which comprises from 0.1 pmol/ml to 500 pmol/ml of myeloperoxidase.

24. The container of Claim 19 which comprises at least 0.01 pmol/ml of eosinophil peroxidase in a liquid carrier.

25. The container of Claim 19 which comprises from 0.1 pmol/ml to 500 pmol/ml of eosinophil peroxidase.

26. The container of Claim 13 which comprises from 100 nmol/ml to 300 μmol/ml chloride.

27. The container of Claim 13 which comprises from 10 nmol/ml to 50 μmol/ml bromide.

28. The container of Claim 13 which comprises from 1 nmol/ml to 5 μ mol/ml iodide.

29. The container of Claim 13 which comprises a peroxide producing oxidase effective to generate from 1 pmol to 50 μ mol peroxide per ml per minute when in the presence of a substrate from the oxidase.

30. The container of Claim 13 which comprises glucose oxidase effective to generate from 1 pmol to 50 μ mol peroxide per ml per minute when in the presence of D-glucose.

31. A hermetically sealed container which comprises, in the substantial absence of oxygen, an air activatable disinfectant-sterilent solution comprising:

(a) from 0.1 pmol/ml to 500 pmol/ml of a haloperoxidase selected from the group consisting of myeloperoxidase, eosinophil peroxidase, lactoperoxidase and combinations thereof;

(b) a halide selected from the group consisting of 100 nmol/ml to 300 μ mol/ml chloride, 10 nmol/ml to 50 μ mol/ml bromide, 1 nmol/ml to 5 μ mol/ml iodide and combinations thereof;

(c) from 0.005 μ mol/ml to 50 μ mol/ml of an α -amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine and the alkyl esters of any members of the group;

(d) from 0.05 units/ml to 10 units/ml of an enzyme capable of oxidizing a substrate and reducing oxygen to hydrogen peroxide; and

(e) from 0.1 μ mol/ml to 10 μ mol/ml of a substrate for the enzyme; in a liquid carrier; and means for releasing the disinfectant-sterilent solution from the container.

32. A hermetically sealed container which comprises, in the substantial absence of oxygen, an air activatable ophthalmic solution comprising:

(a) from 0.1 pmol/ml to 500 pmol/ml of a haloperoxidase selected from the group consisting of myeloperoxidase, eosinophil peroxidase, lactoperoxidase and combinations thereof;

(b) a halide selected from the group consisting of 100 nmol/ml to 300 μ mol/ml chloride, 10 nmol/ml to 50 μ mol/ml bromide, 1 nmol/ml to 5 μ mol/ml iodide and combinations thereof;

(c) from 0.005 $\mu\text{mol/ml}$ to 50 $\mu\text{mol/ml}$ of an α -amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine and the alkyl esters of any members of the group;

~~(d) from 0.05 units/ml to 10 units/ml of an enzyme capable of~~
oxidizing a substrate and reducing oxygen to hydrogen peroxide; and

(e) from 0.1 $\mu\text{mol/ml}$ to 10 $\mu\text{mol/ml}$ of a substrate for the enzyme;
in a liquid carrier; and means for releasing the ophthalmic solution from the container.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/06687

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/02, 9/08; A61K 38/44; A01N 33/00

US CL : 435/189, 192, 264; 424/94.4; 514/579

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/40, 189, 192, 264; 424/94.4; 514/579

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,473,550 A (ROSENBAUM et al.) 25 September 1984, see entire document.	1-32
Y	US 4,588,586 A (KESSLER et al.) 13 May 1986, see entire document.	1-32
A	US 4,937,072 A (KESSLER et al.) 26 June 1990.	1-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* Document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

18 JULY 1996

Date of mailing of the international search report

04 SEP 1996

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06687

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/06687

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS

search terms: robert allen, halide, chloride, bromide, iodide, haloperoxidase, myeloperoxidase, eosinophil peroxidase, lactoperoxidase, aliphatic amino acids, glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, container, canister, enclosure, seal, compartment, yeast, bacteria, microorganism, organism, mold, disinfect, sterilize, ophthalmic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claims 1-12, 21, drawn to method for killing or inhibiting the growth of microorganisms.

Group II, claims 13-20, 22-32, drawn to a hermetically sealed container.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Group I does not use the apparatus of Group II, and is therefore not linked with Group I in a single general inventive concept.